

REMARKS**I. Amendment to the Specification**

The Examiner has objected to the title of the invention as not clearly indicative of the invention to which the claims are directed. The Examiner suggests that the title be amended to recite the cytokine-binding domain of the specific receptors being examined. Applicants have amended the title in accordance with the Examiner's suggestion and respectfully request withdrawal of the objection to the title. No new matter has been added to the specification.

II. Status of the claims

Claims 1-11 and 32-35 were pending in the present application. Claims 1, 2, 4-6, 8, 10, 11, and 32-34 have been amended. Claims 3, 7, 9, and 35 have been canceled. Claims 12-31 have been withdrawn as being drawn to a non-elected invention. Claims 36-40 have been newly added. Accordingly, upon entry of this Amendment, claims 1, 2, 4-6, 8, 10-34, and 36-40 will be pending. No new matter has been added.

Support for the amendments to the claims and the new claims can be found in the specification and claims as originally filed. Support for the term "structure" used in amended claims 1, 2, 4-6, 8, 10, 11, and 32-35, can be found throughout the specification, for example, at p. 2, ll. 21-23 and ll. 30-32.

In particular, support for the amendments to claim 1, can be found in the specification as follows: "human," at pp. 12-13 (human amino acid residues identified in Table 2); "consisting of a portion of D4 β_c said portion defined by a portion of the B'-C' loop of D4 β_c and a groove defined by the B'-C', F'-G' loops and the N-terminal section of D4 β_c ," at p. 2, ll. 30-31, p. 3, ll. 25-27, p. 4, ll. 8-10 and ll. 22-24, and p. 5, ll. 4-6; and "having a surface representation and alignment to a structure according to Figure 1" at p. 5, ll. 12-24 and Figure 1.

Support for the amendments to claim 4 can be found in previous claim 3 (now canceled). Support for the amendments to claims 32-34 can be found in Figure 1C, and at p. 10, ll. 23-30. Support for new claims 36 and 38 can be found in the specification at p. 11, ll. 3-5 and ll. 21-27. Support for new claim 37 can be found in the specification at p. 26, ll. 18-20. Support for new claim 39 can be found in the specification at p. 10, ll. 34-37. Support for new claim 40 can be found in the specification at p. 11, ll. 7-9.

III. Election/Restriction

Applicants thank the Examiner for acknowledging Applicants' election with traverse of Group I (claims 1-11) and, further, for acknowledging that Group II (claims 32-35) will be rejoined with Group I if the allowed process claims are of the same scope as allowable product claims.

IV. Rejection of the claims under 35 U.S.C. § 112, ¶ 1

Claim 1 is rejected under 35 U.S.C. § 112, ¶ 1, as failing to comply with the written description requirement. Specifically, the Examiner contends that the claims contain subject matter that is not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention at the time the application was filed.

Applicants note that the Examiner indicates that the specification, on pages 2, 3, 4, and 5, provides support for the following claim language: "the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4 or an analogous structure" and states that the rejection can be obviated by amending the claims to recite the limitations recited in the specification (Office Action dated June 6, 2005, p. 3, ll. 19-21). Accordingly, Applicants have amended claim 1 to incorporate the language "portion of the B'-C' loop of D4β_c and a groove defined by the B'-C', F'-G' loops and the N-terminal section of D4β_c." Accordingly, Applicants submit that this rejection has been obviated.

Claims 1-6, 8, 10-11, and 32-35 are also rejected under 35 U.S.C. § 112, ¶ 1, as containing subject matter which was not described in the specification in such a way as to reasonably convey to

one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner contends that the specification only sets forth the specific residues 338-438 of D4 β_c expressed using the pEC611 vector in *E. coli* and that an antibody BION-1MoAb was raised against D4 β_c and used to obtain crystals of the antibody and D4 β_c . Further, the Examiner contends that the specification provides no sequence data that might allow one to characterize the claimed domain as human, mouse or another species, and that, because species homologues often display low sequence identity, identification based solely on sequence similarity is impossible. The Examiner further contends that "the structure of the cytokine-binding domain being claimed is not described [t]he skilled artisan cannot envision the detailed structure of the encompassed domain molecules and therefore conception is not achieved until reduction to practice has occurred [and] [t]he nucleic acid itself is required."

Applicants respectfully traverse the rejection. Applicants' invention is the characterization of the structure and function of the portion of the three-dimensional structure of domain 4 of the GM-CSF/IL-3/IL-5 common beta chain (D4 β_c) human receptor involved in cytokine high affinity binding and transduction of cytokine signals through the receptor (specification, p. 2, ll. 18-24). Applicants' characterization of the claimed structure of the human D4 β_c receptor led to development of a monoclonal antibody that is the first common antagonist of the GM-CSF, IL-3, and IL-5 receptors (specification, p. 2, ll. 15-17).

The claims have been amended to clarify that the claimed structure is "human." As disclosed in the specification, based on the known human D4 β_c receptor sequence (specification, for example, pp. 12-13, human amino acid residues identified in Table 2), it would have been readily understood by one having ordinary skill in the art at the time of invention that the disclosed D4 β_c receptor is human. Every reference to a specific amino acid residue recited in the claims and disclosed in the specification corresponds to the amino acid sequence of the known human D4 β_c receptor. The sequence data for the human D4 β_c receptor was well-known by those in the art at the time of the invention (Exhibit 1, European Bioinformatics Institute database detailing the human cytokine receptor sequence, dated August 1, 1991; Exhibit 2, Hayashida et al., *Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF)*:

reconstitution of a high-affinity GM-CSF receptor, PROC. NATL. ACAD. SCI. U.S.A. 87:9655-9659 (1990)). Indeed, comparison of the known human D4 β_c sequence with the disclosed and claimed amino acid residues shows that Applicants' disclosure is based on examples using the human D4 β_c sequence as disclosed in Exhibits 1 and 2.

Applicants are under no obligation to include the known human D4 β_c receptor sequence in the body of the specification (*Capon v. Eshhar*,¹ 2005 U.S. App. LEXIS 16865, *17 and *23 (Fed. Cir. 2005) (concluding that the Board of Patent Appeals and Interferences erred in determining "that 'controlling precedent' required inclusion in the specification of the complete nucleotide sequence," stating "[w]hen the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh"); see MPEP § 2163, subsections 2 and 3(a) (referring to *Hybridtech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80 and 1384 (Fed. Cir. 1986)) for the proposition that "[i]formation which is well known in the art need not be described in detail in the specification" and that "[t]he description need only describe in detail that which is new or not conventional"). Accordingly, one having ordinary skill in the art at the time of the invention would have understood that Applicants had possessed the claimed invention.

Applicants respectfully disagree with the Examiner's contention that "the structure of the cytokine-binding domain being claimed is not described . . . [t]he skilled artisan cannot envision the detailed structure of the encompassed domain molecules and therefore conception is not achieved until reduction to practice has occurred . . . [and] [t]he nucleic acid itself is required." Applicants submit that the claimed structure is set forth in detail in Figures 1-9, and in the specification at pp. 9-12, including Tables 1 and 2, and would be readily recognized as corresponding to the human D4 β_c receptor sequence based on the specified amino acid residues referred to throughout the specification and the claims which correspond to the human D4 β_c sequence. Applicants submit that the Figures and the written disclosure of the claimed structure in light of the known and readily available human D4 β_c sequence demonstrate possession of the claimed invention.

¹ Available at: <http://fedcir.gov/opinions/03-1480.pdf> (quotes on pp. 11 and 12).

Further, Applicants submit that the amendments to the claims to recite “human” and “structure” obviate the Examiner’s remaining rejections to the claims under 35 U.S.C. § 112, ¶ 1, for at least those reasons provided above. Specifically, Applicants submit that the amendments to the claims obviate the Examiner’s rejection based on alleged failure to identify whether the claimed D4β_c receptor sequence is human, mouse or other species. To this end, Applicants take note of the Examiner’s observation that the “species homologues often display low sequence identity so that identification based solely on sequence similarity is impossible.” To the contrary, Applicants submit that it is, in part, for this reason that one having skill in the art would readily recognize that the claimed D4β_c receptor sequence is human as the specified amino acid residues are drawn by number to the corresponding amino acid sequence of the claimed D4β_c human receptor.

Applicants respectfully request that the rejection be withdrawn.

V. Rejection of the claims under 35 U.S.C. § 112, ¶ 2

Claims 1-6, 8, 10-11, and 32-35 are rejected under 35 U.S.C. § 112, ¶ 2 as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Specifically, the Examiner contends that claims 1, 2, 4, 6, and 32-35 are vague and indefinite because no sequence identification is recited in the claims, and declares that “[r]ecitation of these residues in the claim are meaningless in the absence of a reference amino acid sequence” (Office Action dated June 6, 2005, p. 7, ll. 16-17). The Examiner further contends that the terms “tyrosine residue” in claim 3, “including” in claims 1, 3, and 6, “having” in claim 32, and the phrase “β_c chain is derived from a receptor” are unclear. Finally, the Examiner contends that each of claims 32-35 is improperly dependent on a method claim, and that claims 5, 8, and 10-11 are vague and indefinite insofar as they depend on the above claims for their limitations.

Applicants respectfully traverse the Examiner’s rejection. As noted above in relation to the arguments submitted in response to the rejections under 35 U.S.C. § 112, ¶ 1, the claims have been amended to recite a “human” D4β_c receptor. The corresponding human sequence information was known by those of skill in the art and readily available to the skilled artisan since at least 1990

(Exhibits 1 and 2). Thus, Applicants submit that the claims are clear and definite. Furthermore, Applicants submit that amendments to the claims 1, 3, 6, 10, and 32-35 obviate the specific rejections made in relation to each of these claims. Accordingly, Applicants respectfully request that the rejection of the claims be withdrawn.

VI. Rejection of the claims under 35 U.S.C. § 102(b)

Claims 1-6, 8, 9, 10-11, 32-35 are rejected as being anticipated by the WO 97/07215 ('215) patent. The Examiner contends that the reference discloses an isolated β_c chain of the IL-3 receptor (β_c Domain) which binds to at least one cytokine selected from GM-CSF, IL-3 and IL-5, the production of antibodies via immunization with fragments of the β_4 Domain, and the isolation of short peptides of the β_4 Domain.

Applicants respectfully traverse the rejection. The claims have been amended to recite "a portion of D4 β_c said portion defined by a portion of the B'-C' loop of D4 β_c and a groove defined by the B'-C', F'-G' loops and the N-terminal section of D4 β_c ." Applicants respectfully submit that the amendments to the claims, which recite that the claimed structure is "a portion of D4 β_c " and distinguish the claimed invention over the prior art. Specifically, the common meaning of "portion" is "a section or quantity within a larger thing; a part of a whole."² Further, the term "domain," as applied to the cytokine receptor would itself be understood to be a region of the cytokine receptor—not the whole receptor.³

The cited prior art does not disclose, expressly or inherently, the claimed three-dimensional region (identified as loops and grooves) of the portion of the D4 β_c essential for defining the domain area which binds to at least one cytokine that is capable of transducing a cytokine signal through a receptor. Therefore, the cited art fails to teach or suggest each and every

² Available at: <http://dictionary.reference.com/search?q=portion> (last visited October 3, 2005).

³ Domain is defined as "any of the three-dimensional subunits of a protein that together make up its tertiary structure, that are formed by folding its linear peptide chain, and that are variously considered to be the basic units of protein structure, function, and evolution." Definition of "domain" available at <http://dictionary.reference.com/search?q=domain> (last visited October 3, 2005).

aspect of the claimed invention. Accordingly, Applicants respectfully request that the Examiner withdraw the above rejection.

Claims 1-6, 8, 9, 10-11, and 32-35 are rejected under 35 U.S.C. § 102(b) as being anticipated by Woodcock et al. (Woodcock). Specifically, the Examiner contends that the reference discloses using specific antibodies which immunoprecipitate the β_c chain of the IL-3 receptor (β_4 Domain) which binds to at least one cytokine selected from GM-CSF, IL-3 and IL-5 and for affinity purification of the β_c region.

The present invention distinguishes over the prior art for at least those reasons noted in relation to the '215 patent noted above. Woodcock does not teach or suggest the structure of the specific portion of the D4 β_c essential for defining the domain area which binds to at least one cytokine that is capable of transducing a cytokine signal through a receptor. Accordingly, Applicants respectfully request that the Examiner withdraw the rejection.

CONCLUSION

Applicants respectfully request entry of the foregoing amendments and remarks in the file history of this application. In view of the above amendments, Applicants believe the pending application is in condition for allowance. The Examiner is invited to contact the undersigned with any questions or to resolve any points so as to facilitate allowance. Reconsideration of the pending application and allowance of the claims is respectfully solicited.

Dated: October 6, 2005

Respectfully submitted,

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XX
SV   M59941.1
XX
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DT   01-JUL-1999 (Rel. 60, Last updated, Version 3)
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XX
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KW   lymphokine receptor.
XX
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RA   Hayashida K., Kitamura T., Gorman D.M., Arai K., Yokota T., Miyajima A.;
RT   "Molecular cloning of a second subunit of the receptor for human
RT   granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution
RT   of a high-affinity GM-CSF receptor";
RL   Proc. Natl. Acad. Sci. U.S.A. 87(24):9655-9659(1990).
XX
RN   [2]
RP   1-2996
RA   Kitamura T.;
RT   ;
RL   Submitted (06-FEB-1991) to the EMBL/GenBank/DDBJ databases.
RL   Toshio Kitamura, Department of Molecular Biology, DNAX Research Institute
RL   of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA
RL   94304-1104, USA
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XX
XX
SQ

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Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor

(cytokine receptor/hemopoietic growth factor/hemopoiesis gene family)

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Communicated by George Palade, September 7, 1990

ABSTRACT Using the mouse interleukin 3 (IL-3) receptor cDNA as a probe, we obtained a homologous cDNA (KH97) from a cDNA library of a human hemopoietic cell line, TF-1. The protein encoded by the KH97 cDNA has 56% amino acid sequence identity with the mouse IL-3 receptor and retains features common to the family of cytokine receptors. Fibroblasts transfected with the KH97 cDNA expressed a protein of 120 kDa but did not bind any human cytokines, including IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Interestingly, cotransfection of cDNAs for KH97 and the low-affinity human GM-CSF receptor in fibroblasts resulted in formation of a high-affinity receptor for GM-CSF. The dissociation rate of GM-CSF from the reconstituted high-affinity receptor was slower than that from the low-affinity site, whereas the association rate was unchanged. Cross-linking of ¹²⁵I-labeled GM-CSF to fibroblasts cotransfected with both cDNAs revealed the same cross-linking patterns as in TF-1 cells—i.e., two major proteins of 80 and 120 kDa which correspond to the low-affinity GM-CSF receptor and the KH97 protein, respectively. These results indicate that the high-affinity GM-CSF receptor is composed of at least two components in a manner analogous to the IL-2 receptor. We therefore propose to designate the low-affinity GM-CSF receptor and the KH97 protein as the α and β subunits of the GM-CSF receptor, respectively.

All hemopoietic cells ultimately arise from self-renewing pluripotent hemopoietic stem cells which are produced continuously in the bone marrow. Bone marrow stromal cells and a number of soluble factors, known as cytokines, play crucial roles in this process. Among these cytokines, interleukin 3 (IL-3), also known as multi-colony-stimulating factor (multi-CSF), stimulates early progenitor cells and supports the development of various cell lineages (1). While GM-CSF was initially defined as a factor that gives rise to granulocyte and macrophage colonies *in vitro*, recent evidence indicates that GM-CSF has broader biological activities, including stimulation of early hemopoietic progenitor cells and the development of other cell lineages (2, 3).

The biological effects of both GM-CSF and IL-3 are mediated by specific cell surface receptors. The human GM-CSF (hGM-CSF) receptor, cloned by Gearing *et al.* (4), exhibits low-affinity binding for GM-CSF when expressed on COS7 cells. Although there is evidence indicating that GM-CSF induces tyrosine phosphorylation (5), no tyrosine kinase consensus sequence was found (4). It is likely that the functional high-affinity GM-CSF receptor is composed of multiple subunits. Both GM-CSF and IL-3 induce tyrosine

phosphorylation of a similar set of proteins (6–8) and they have overlapping biological activities (1–3). In addition, evidence indicates that the binding of hGM-CSF to its receptor is partially inhibited by human IL-3 (hIL-3) and vice versa (9–11). These results suggest that the hGM-CSF receptor and the hIL-3 receptor may share a common component.

Mouse IL-3 (mIL-3)-responsive cells express low- and high-affinity receptors for IL-3 (12–14). We recently isolated a cDNA (AIC2A) encoding a low-affinity mIL-3 binding protein which is a member of a recently identified cytokine receptor family (15). Although AIC2A does not contain a tyrosine kinase consensus sequence, AIC2A is a component of the high-affinity receptor (J. Schreurs and A.M., unpublished results). We also isolated a cDNA (AIC2B) which is highly identical (95% at the nucleotide level) to the IL-3 receptor cDNA (AIC2A) but is derived from a distinct gene (16). Despite its unusually high sequence identity with the IL-3 receptor, the AIC2B protein does not bind IL-3 and its function is currently unknown.

In this report, we present the cloning of a human cDNA which has homology with the mIL-3 receptor cDNA.[§] The protein encoded by the cloned cDNA alone did not bind any of the cytokines tested. However, it conferred high-affinity binding for hGM-CSF when cotransfected with the low-affinity hGM-CSF receptor cDNA. This result indicates that the cloned IL-3 receptor-like cDNA (KH97) encodes a second subunit of the high-affinity hGM-CSF receptor.

MATERIALS AND METHODS

Construction of cDNA Library and Isolation of cDNA Clones. Poly(A)⁺ RNA isolated from TF-1 cells (17) was converted to double-stranded cDNA by using oligo(dT) primers or specific primers corresponding to the cDNA sequence (Fig. 1). cDNA libraries were constructed either in the λ gt11 phage vector or the simian virus 40-based mammalian expression vector pMB18 (K. Maruyama and A.M., unpublished results). Using a ³²P-labeled mouse IL-3 receptor cDNA fragment (15) as a hybridization probe, we isolated a 3-kilobase (kb) human cDNA fragment (KH85) from the phage library under low-stringency conditions: hybridization at 42°C with 6× SSPE (1× SSPE is 150 mM NaCl/100 mM NaH₂PO₄/1 mM EDTA, pH 7.4) in the presence of 20% (vol/vol) formamide and washing at 50°C with 2× SSPE.

Abbreviations: IL-2, IL-3, etc., interleukin 2, interleukin 3, etc.; GM-CSF, granulocyte-macrophage colony-stimulating factor; h-, human; m-, mouse.

[‡]To whom reprint requests should be addressed.

[§]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38275).

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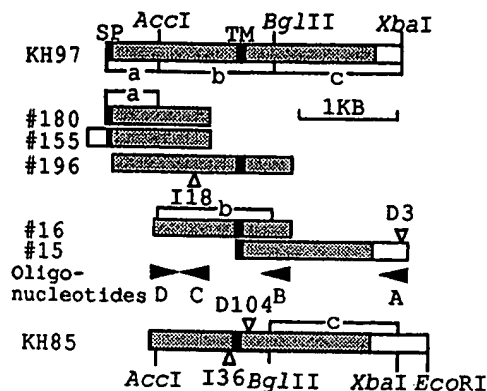


Fig. 1. The cloned cDNA fragments. Typical cDNA fragments obtained by using specific oligonucleotide primers are shown as bars, with the unshaded portion indicating the noncoding regions. The locations of oligonucleotides used to prime the cDNA synthesis are shown as arrowheads A, B, and C. An oligonucleotide corresponding to the region D and the KH85 cDNA fragment were used to isolate these cDNA fragments. All the cloned DNA fragments had the same sequence except for small insertions and deletions as indicated by I18 (an 18-base-pair insertion), I36 (a 36-base-pair insertion), D104 (a 104-base-pair deletion), and D3 (a 3-base-pair deletion). The KH97 cDNA was constructed with #180 (fragment a), #16 (fragment b), and KH85 (fragment c) as indicated. SP and TM indicate the signal peptide and the transmembrane domain, respectively. Scale indicates 1 kilobase.

The cDNA encoding the low-affinity hGM-CSF receptor was isolated by using the polymerase chain reaction primed with specific oligonucleotides corresponding to the 5' untranslated and the 3' untranslated regions of the published sequence (4). The cloned DNA fragment was sequenced to confirm the identity of the cDNA.

Transfection. Five micrograms of either individual plasmid DNA or a combination of two plasmid DNAs was transfected into semiconfluent COS-7 cells (African green monkey kidney cells expressing the T antigen of simian virus 40) by the DEAE-dextran method as described previously (18). Three days after transfection, COS-7 cells were harvested and analyzed by ligand binding assays or chemical cross-linking

experiments. NIH 3T3 mouse cells were stably transfected, using the *neo* gene as a selection marker, by the calcium phosphate procedure (19). Stable transfectants were selected with G418 at 1 mg/ml.

Radiolabeling of hGM-CSF and Binding Experiments. *Escherichia coli*-derived hGM-CSF iodinated with Bolton-Hunter reagent (125 I-GM-CSF) was used for binding assays as described previously (20). Dissociation constants were obtained by the LIGAND program (21). Chemical cross-linking was performed with 0.2 mM disuccinimidyl suberate on transfected COS-7 cells (10^6 cells) or NIH 3T3 stable transfectants (3×10^6 cells) preincubated with 4 nM 125 I-GM-CSF. Proteins were analyzed as described previously (20).

RESULTS

Isolation and Characterization of a Human cDNA Homologous to the mIL-3 Receptor cDNA. Using the mIL-3 receptor cDNA as a probe, we screened a cDNA library made from a human erythroleukemic cell line, TF-1, which responds to multiple human factors, including IL-3, IL-4, IL-5, GM-CSF, and erythropoietin (17). A cDNA clone (KH85) homologous to the mIL-3 receptor cDNA (approximately 70% identical at the nucleotide level) was obtained from about 4×10^5 independent clones. This clone lacks about 600 bases from its 5' end compared with the sequence of the mouse cDNA. We therefore prepared cDNA libraries by using specific primers based on the KH85 sequence and screened these libraries with the KH85 probes (Fig. 1). We found only one type of cDNA among 26 positive clones analyzed. Although several cDNAs with an insertion and/or a deletion were isolated (Fig. 1), these cDNAs seemed to be created by alternative splicing rather than encoded by a distinct gene, because the insertions and deletions were found at sites corresponding to the exon-intron junctions of the mouse AIC2 genes (D.M.G., unpublished results). We reconstructed a cDNA (KH97) encoding the entire protein (Fig. 1) and used this for further studies.

Comparison of the amino acid sequence encoded by the KH97 cDNA with that of the AIC2A and AIC2B proteins showed 56% and 55% identity, respectively (Fig. 2). The homology was distributed throughout the coding region in

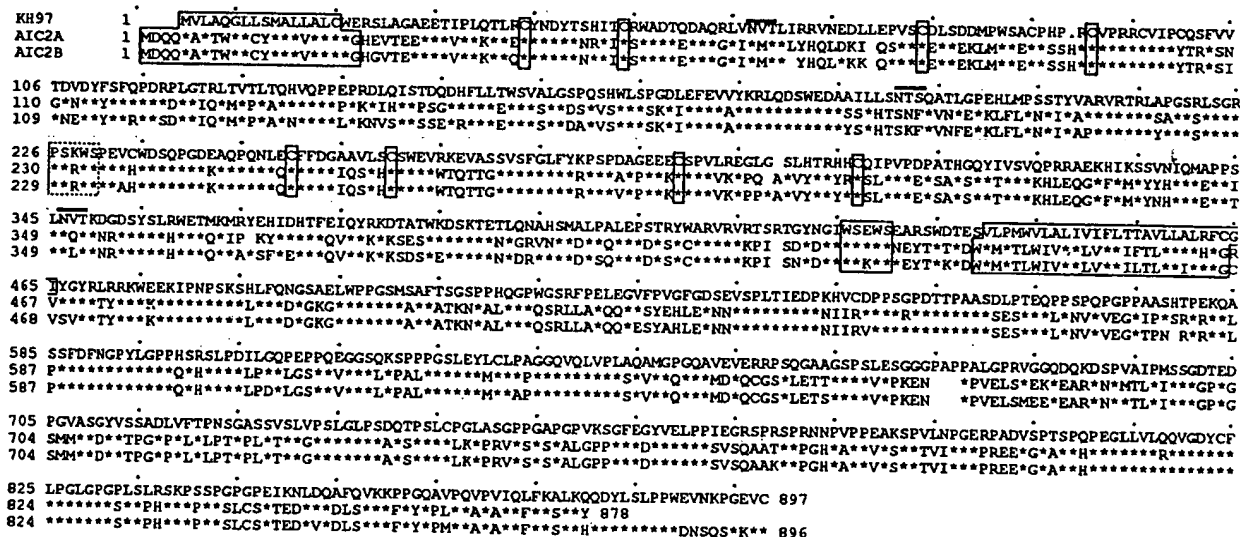


Fig. 2. Comparison of amino acid sequence of KH97 with the mouse AIC2A and AIC2B proteins. Asterisks on the mouse AIC2A and AIC2B sequences indicate the same amino acid as that of the human KH97 sequence. The signal sequences and the transmembrane domains are shown by boxes. The conserved cysteine residues and the WSXWS motif of the cytokine receptor family are also indicated by boxes. The WSXWS-like sequence is indicated by the box with the dotted outlines. Potential N-linked glycosylation sites are marked by bars.

both cases. The AIC2B protein has an extra 18 amino acids at the C terminus compared with the mIL-3 receptor (AIC2A), and the KH97 protein also has the extra 18 amino acids (Fig. 2). Because of the high sequence similarity between AIC2A and AIC2B (16), it is not clear to which of the mouse genes the KH97 cDNA corresponds.

The KH97 mRNA was detected in the myelogenous leukemic cell lines TF-1 and KG1 but not in the NK cell line YT or the mouse IL-3- and GM-CSF-dependent cell line PT18 under stringent hybridization conditions (Fig. 3).

Expression of KH97 in COS-7. A transient expression system using COS-7 cells was used to evaluate expression of the KH97 cDNA. Because of its extensive sequence similarity to the mIL-3 receptor cDNA, we examined whether hIL-3 could bind to COS-7 cells transfected with the KH97 cDNA. Using up to 20 nM 125 I-hIL-3, we could not detect any specific binding. We also constructed full-length cDNAs of KH97 variants (Fig. 1), which were presumably derived from alternative splicing, and tested the binding of hIL-3 to transfected COS-7 cells. However, we did not find any specific binding. We then examined the binding of other cytokines. However, hIL-2 (1 nM), hIL-4 (1 nM), hIL-5 (5 nM), hGM-CSF (20 nM), and human erythropoietin (10 nM) all showed no specific binding to KH97-transfected COS-7 cells at the indicated concentrations. To exclude the possibility that COS-7 cells failed to express the protein encoded by the KH97 cDNA, we prepared antibodies against a peptide encoded by the KH97 cDNA and used them to detect the KH97 cDNA-encoded protein in COS-7 cells. Western blotting using anti-peptide antibodies confirmed the expression of a 120-kDa protein in the KH97 cDNA-transfected COS-7 cells (data not shown). We therefore concluded that the KH97 protein did not bind any cytokines examined when expressed in COS-7 cells.

Cotransfection of the KH97 cDNA and the GM-CSF Receptor cDNA. Although the KH97 protein did not bind any of the cytokines we examined, there still remained the possibility that it is a component of another known or unknown cytokine receptor. It has been shown that the β chain of the IL-2 receptor does not bind IL-2 when expressed in COS-7 cells, but it does bind IL-2 with intermediate affinity in Jurkat cells (22). Furthermore, coexpression of the low-affinity IL-2 receptor (α chain) and the β chain forms a high-affinity binding site (22). These observations indicate that the binding of a ligand to its receptor can be determined by the interaction of multiple proteins. Combining this idea with the observa-

tion that the IL-3 and GM-CSF receptors may share a common element (9–11), we considered the possibility that the KH97 protein is a subunit of the hGM-CSF receptor. To examine this possibility, we cotransfected the KH97 cDNA with the low-affinity GM-CSF receptor cDNA (4).

We examined the equilibrium binding of hGM-CSF to COS-7 cells transfected with the hGM-CSF receptor cDNA, the KH97 cDNA, or a combination of these cDNAs (Fig. 4). The hGM-CSF receptor expressed exclusively low-affinity binding sites ($K_d = 3.2$ nM) as reported by Gearing *et al.* (4), whereas the KH97 protein alone did not express any detectable binding for GM-CSF. However, cotransfection of these two cDNAs resulted in the expression of both high- (120 pM) and low- (6.6 nM) affinity binding sites.

The same results were obtained with stable transfectants of NIH 3T3 cells (Fig. 4): hGM-CSF receptor cDNA-transfected NIH 3T3 cells bound hGM-CSF with $K_d = 2.7$ nM, whereas the NIH 3T3 transfected with both the hGM-CSF receptor and the KH97 cDNAs bound hGM-CSF with $K_d = 170$ pM. Because of the low expression of the low-affinity hGM-CSF receptor compared with the KH97 protein in this NIH 3T3 transfectant, no statistically significant low-affinity binding site was found by using the LIGAND program (21). Again no specific binding of hGM-CSF was detected in the cells transfected with the KH97 cDNA alone.

In both COS-7 and NIH 3T3 cells, binding of 125 I-hGM-CSF was blocked by hGM-CSF but not by hIL-3. In addition, cotransfection of the KH97 cDNA with the human IL-2 receptor α chain cDNA or the human IL-4 receptor cDNA did not change the binding affinity of their respective ligands. Cotransfection of the cDNAs encoding the low-affinity hGM-CSF receptor with that for the IL-2 receptor β chain, the IL-4 receptor, mouse AIC2A, or AIC2B also did not change the affinity for hGM-CSF. Thus, formation of the high-affinity GM-CSF receptor is specific to the combination of the low-affinity GM-CSF receptor and the KH97 protein.

We then analyzed the binding kinetics to determine whether the high-affinity binding of hGM-CSF to cotransfected cells was due to an increased rate of association or a decreased rate of dissociation. As shown in Fig. 5, GM-CSF

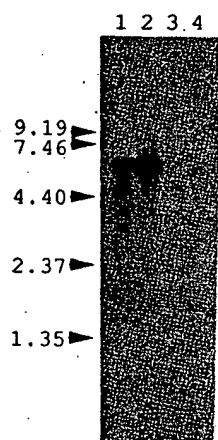


FIG. 3. Northern blotting of poly(A)⁺ RNA from various cell lines. Three micrograms of each poly(A)⁺ RNA was electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized with the 32 P-labeled KH97 cDNA. Lane 1, TF-1; lane 2, KG-1; lane 3, YT; and lane 4, PT18. Sizes are given in kb.

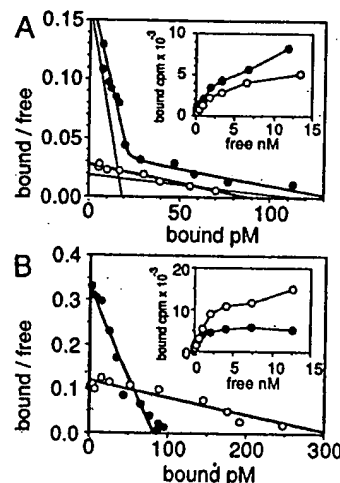


FIG. 4. Binding of 125 I-hGM-CSF. (A) Duplicate suspensions of 2×10^5 COS-7 cells transiently transfected with the hGM-CSF receptor and KH97 cDNAs were used for 125 I-hGM-CSF binding assays. (B) Duplicate suspensions of NIH 3T3 stable transfectants (5×10^6 cells) were used for binding assays. Binding assays were performed at 4°C. O, Cells transfected with the hGM-CSF receptor cDNA and the vector DNA. ●, Cells cotransfected with the hGM-CSF receptor cDNA and the KH97 cDNA. Scatchard plots of the binding data are shown. (Insets) Equilibrium binding profiles.

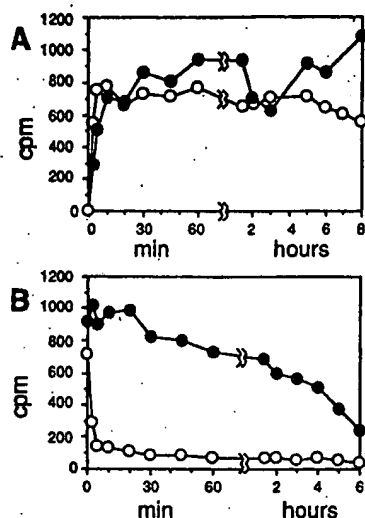


Fig. 5. Kinetics of binding of ¹²⁵I-hGM-CSF to the receptor. (A) Association rate. NIH 3T3 stable transfectants were incubated with 200 pM ¹²⁵I-hGM-CSF at 4°C for various times and the cell-bound radioactivity was measured. (B) Dissociation rate. A 200-fold excess of unlabeled hGM-CSF was added to the NIH 3T3 stable transfectants, which were preincubated with 200 pM ¹²⁵I-hGM-CSF for 4 hr at 4°C, and the residual cell-bound radioactivity was then measured at various times at 4°C. ○, NIH 3T3 cells expressing the low-affinity hGM-CSF receptor; ●, NIH 3T3 cells expressing both the hGM-CSF receptor and the KH97 protein.

binding increased with similar kinetics in both hGM-CSF receptor cDNA-transfected cells and cotransfected cells. In dissociation experiments, the addition of unlabeled hGM-CSF to cells pre-equilibrated with ¹²⁵I-hGM-CSF led to the rapid release of radioligand from the hGM-CSF receptor-transfectants ($t_{1/2}$ = 2 min), and a slow release ($t_{1/2}$ = 290 min) from the cells expressing both proteins. These results indicate that the high-affinity binding of hGM-CSF to the co-transfected cells is due to the slow dissociation of hGM-CSF from the receptor.

Cross-Linking of hGM-CSF to the KH97 Protein. To test if the KH97 protein actually binds hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor in COS-7 or NIH 3T3 cells, we performed chemical cross-linking experiments using ¹²⁵I-hGM-CSF (Fig. 6). Cross-linking of ¹²⁵I-hGM-CSF to the low-affinity hGM-CSF receptor-expressing cells showed only one band of 95 kDa (Fig. 6B, lane 5), whereas no specific band was detected in cells expressing only the KH97 protein (Fig. 6B, lane 9). However, cross-linking of the cells expressing both proteins revealed three bands, of 95, 135, and 210 kDa (Fig. 6B, lane 6). This cross-linking pattern was identical to that obtained with TF-1 cells (Fig. 6B, lane 10). These bands were not detected when cross-linking was performed in the presence of an excess of unlabeled hGM-CSF (data not shown). Subtraction of the molecular mass of hGM-CSF from the molecular mass of the cross-linked proteins results in calculated masses of 80, 120, and 195 kDa, which correspond, respectively, to the low-affinity hGM-CSF receptor, the KH97 protein, and possibly a complex of the two. Similar results were obtained with NIH 3T3 transfectants (Fig. 6B, lanes 1–4).

Cross-linking of hGM-CSF to the KH97 protein was further confirmed by making cytoplasmic domain deletion mutants of KH97 (Fig. 6A). Deletion mutants of the KH97 cDNA were cotransfected with the low-affinity hGM-CSF receptor cDNA into COS-7 cells. Whereas the band at 95 kDa was not changed, the band at 135 kDa was shifted to lower molecular masses in cells transfected with these deletion mutants (Fig.

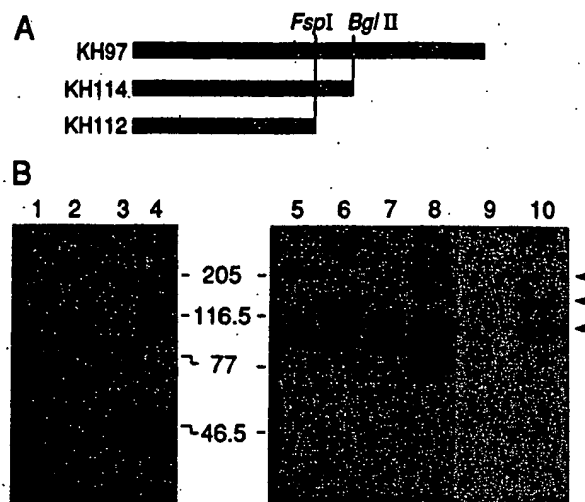


Fig. 6. Expression of GM-CSF receptors on COS-7 cells and NIH 3T3 cells. (A) Structure of the cDNAs encoding the KH97 protein and its deletion mutants. KH114 is truncated at the Bgl II site and KH112 is truncated at the Fsp I site (Fig. 1). (B) Cross-linking of ¹²⁵I-hGM-CSF to the transfected COS-7 cells or NIH 3T3 stable transfectants. Lanes 1–4, NIH 3T3 cells; lanes 5–9, COS-7 cells. Cells were transfected with the following: lane 1, mock transfection; lane 2, GM-CSF receptor cDNA; lane 3, KH97 cDNA; lane 4, GM-CSF receptor cDNA and KH97 cDNA; lane 5, GM-CSF receptor cDNA; lane 6, GM-CSF receptor cDNA and KH97 cDNA; lane 7, GM-CSF receptor cDNA and KH114 cDNA; lane 8, GM-CSF receptor cDNA and KH112 cDNA; and lane 9, KH97 cDNA. Lane 10, cross-linking with TF-1 cells. Specific bands detected in COS-7 cells transfected with deletion mutants are marked by dots (lanes 6–8). Protein masses are given in kDa.

6, lanes 6–8, marked by dots). The shift of the molecular mass was consistent with the shift of the bands revealed by Western blotting using anti-peptide antibodies against the KH97 protein (data not shown). These results clearly indicate that the KH97 protein is cross-linked with hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor. In addition, this experiment demonstrates that formation of the high-affinity receptor for hGM-CSF does not require the cytoplasmic domain of the KH97 protein.

DISCUSSION

Our results demonstrate that the low-affinity hGM-CSF receptor together with the KH97 protein forms a high-affinity receptor for hGM-CSF. This is analogous to the formation of a high-affinity receptor for IL-2 by coexpression of the α and β chains of the IL-2 receptor. The α chain of the IL-2 receptor binds IL-2 with low affinity (23, 24), whereas the β chain by itself does not bind IL-2 when expressed in COS-7 cells (22). However, coexpression of both chains leads to the formation of a high-affinity receptor, and both chains can be cross-linked with IL-2 (22). Because of similarity between the IL-2 and the hGM-CSF high-affinity receptors, we propose to designate the low-affinity hGM-CSF receptor as the α chain and the KH97 protein as the β chain of the hGM-CSF receptor.

It is of particular interest that the KH97 cDNA was isolated on the basis of its homology to the mIL-3 receptor. Among various cytokines the sequence conservation of IL-3 between mouse and human is unusually weak (only 29% identity at the amino acid level) (25). Nevertheless we were able to isolate a human cDNA which was homologous (56% identity at the amino acid level) to the mIL-3 receptor cDNA. However, as described above, we were unable to demonstrate IL-3 bind-

ing to the KH97 protein expressed on fibroblasts. Recently, we isolated a second mouse gene (AIC2B) which is highly homologous to the mIL-3 receptor gene (AIC2A), but the AIC2B protein does not bind mIL-3 (16). One possibility is that the KH97 protein is the human protein corresponding to the mouse AIC2B protein and therefore cannot bind IL-3. If this is the case, there may exist an AIC2A-like human protein which binds hIL-3. To address this question we have extensively searched for additional cDNA clones which might hybridize with either the mouse AIC2A or the human KH97 cDNA probe. However, we could not find any cDNA that is homologous to, yet distinct from, the KH97 cDNA. Since the abundance of the AIC2B mRNA is generally higher than that of the AIC2A (mIL-3 receptor) mRNA in mouse (16), the failure to identify the hIL-3 receptor cDNA may be due to its low abundance.

Another possibility is that, unlike mice, humans do not have two homologous genes. In this case, the IL-3 binding protein, like IL-3, may have only weak conservation between human and mouse. Alternatively, the KH97 protein may be a component of both the hIL-3 and hGM-CSF receptors. IL-3 and GM-CSF induce tyrosine phosphorylation of similar sets of proteins (5) and they have overlapping biological activities (1-3). In addition, binding of hGM-CSF to its receptor is partially blocked by hIL-3 and vice versa (9-11), although IL-3 and GM-CSF have no structural homology. The KH97 protein may be shared between the hIL-3 receptor and the hGM-CSF receptor—i.e., the KH97 protein forms the high-affinity receptor for hGM-CSF with the α chain of the hGM-CSF receptor and it also forms the high-affinity receptor for hIL-3 with an unidentified protein which may or may not bind hIL-3 by itself. If this is the case, the cross-competition as well as overlapping biological activities of the two factors may be explained. It is of interest that no cross-competition of binding between mIL-3 and mGM-CSF has yet been reported. If the AIC2A protein forms the high-affinity mIL-3 receptor with an unidentified protein and the AIC2B protein is the β chain of the mGM-CSF receptor, then there may be no cross-competition between mouse factors. As the mouse low-affinity receptor for GM-CSF has not yet been isolated, at present we are not able to test this hypothesis by using cotransfection with either the AIC2A or the AIC2B cDNA. We have examined the possibility that the low-affinity hGM-CSF receptor may form a high-affinity receptor with either the mouse AIC2A or the AIC2B protein; however, none of these combinations resulted in high-affinity binding with either mouse or human GM-CSF. If there is another human AIC2 homologue which binds hIL-3 in a manner analogous to mouse AIC2A, cross-competition between hIL-3 and hGM-CSF may occur due to another shared component. If this component is present abundantly in mouse, cross-competition may not be observed. In any case, it is important to find whether humans have two AIC2 homologues and also whether mouse AIC2B is the β chain of the mGM-CSF receptor.

Neither the α nor the β chain of the GM-CSF receptor has a tyrosine kinase consensus sequence, and GM-CSF did not induce tyrosine phosphorylation in the NIH 3T3 transfectants expressing the α and β subunits of the GM-CSF receptor (T.K., unpublished data), yet GM-CSF induces tyrosine phosphorylation in hemopoietic cells (5). Thus, signal transduction through the GM-CSF receptor must require additional component(s). To understand the molecular mechanisms of signal transduction it is of particular importance to identify those additional component(s) required for signal transduction.

During the preparation of this manuscript, Metcalf *et al.* (26) reported that transfection of the α subunit of hGM-CSF receptor cDNA in a mGM-CSF-dependent mouse cell line resulted in only low-affinity binding for hGM-CSF and a high

concentration of hGM-CSF stimulated proliferation. Inability of the human α subunit to form a high-affinity receptor in mouse cells is consistent with our result that cotransfection of the human α subunit cDNA and the mouse AIC2A or AIC2B cDNA did not result in a high-affinity binding for hGM-CSF in COS-7 cells. However, the mechanism by which the low-affinity hGM-CSF receptor transmits a growth signal remains unclear.

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